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A Novel Mutation in the Switch 3 Region of $G_s\alpha$ in a Patient with Albright Hereditary Osteodystrophy Impairs GDP Binding and Receptor Activation*

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Albright hereditary osteodystrophy (AHO), a disorder characterized by skeletal abnormalities and obesity, is associated with heterozygous inactivating mutations in the gene for $G_s\alpha$. A novel $G_s\alpha$ mutation encoding the substitution of tryptophan for a nonconserved arginine within the switch 3 region ($G_s\alpha$ R258W) was identified in an AHO patient. Although reverse transcription-polymerase chain reaction studies demonstrated that mRNA expression from wild type and mutant alleles was similar, $G_s\alpha$ expression in erythrocyte membranes from the affected patient was reduced by 50%. A $G_s\alpha$ R258W cDNA, as well as one with arginine replaced by alanine ($G_s\alpha$ R258A), was generated, and the biochemical properties of *in vitro* transcription/translation products were examined. When reconstituted with cyc$^-$ membranes, both mutant proteins were able to stimulate adenylyl cyclase normally in the presence of guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) but had decreased ability in the presence of isoproterenol or AlF_4^- (a mixture of 10 μM $AlCl_3$ and 10 mM NaF). The ability of each mutant to bind and be activated by GTPγS or AlF_4^- was assessed by trypsin protection assays. Both mutants were protected normally by GTPγS but showed reduced protection in the presence of AlF_4^-. The addition of excess GDP (2 mM) was able to rescue the ability of AlF_4^- to protect the mutants, suggesting that they might have reduced affinity for GDP. A $G_s\alpha$ R258A mutant purified from *Escherichia coli* had decreased affinity for GDP and an apparent rate of GDP release that was 10-fold greater than that of wild type $G_s\alpha$. Sucrose density gradient analysis demonstrated that both $G_s\alpha$ R258W and $G_s\alpha$ R258A were thermolabile at higher temperatures and that denaturation of both mutants was prevented by the presence of 0.1 mM GTPγS or 2 mM GDP. The crystal structure of $G_s\alpha$ demonstrates that Arg^{258} interacts with a conserved residue in the helical domain (Gln^{170}). Arg^{258} substitutions would be predicted to open the cleft between the GTPase and helical domains, allowing for increased GDP release in the inactive state, resulting in enhanced thermolability and reduced AlF_4^--induced adenylyl cyclase stimulation and trypsin protection, since activation by AlF_4^- requires bound GDP.

Heterotrimeric guanine nucleotide-binding proteins (G proteins)[1] couple heptahelical receptors to intracellular effectors and are composed of three subunits (α, β, and γ), which are the products of separate genes (reviewed in Refs. 1–3). Each G protein is defined by its α-subunit, which binds guanine nucleotide and couples to downstream effectors such as adenylyl cyclase and phospholipase C. The inactive GDP-bound α-subunit is associated with a tightly but noncovalently bound βγ-dimer. Upon activation by receptor, the α-subunit undergoes a conformational change resulting in release of GDP with concomitant binding of GTP and dissociation from βγ. The GTP-bound α-subunit can then interact with and modulate specific effector enzymes or ion channels. For G_s, these include the stimulation of adenylyl cyclase and modulation of ion channels (4, 5). Hydrolysis of bound GTP to GDP by an intrinsic GTPase activity returns the α-subunit to the inactive state. G protein α-subunits can also be activated by incubation with GTPγS, a nonhydrolyzable GTP analogue, or AlF_4^-, which binds to GDP-bound α-subunits and mimics the γ-phosphate of GTP.

X-ray crystal structures of two G protein α-subunits (transducin and Gi1α) reveal that α-subunits have two domains, a *ras*-like GTPase domain encoding the structural elements for guanine nucleotide binding and effector interaction and a highly helical domain that may be critical to prevent release of GDP in the inactive state (6–12). The guanine nucleotide resides in a cleft between the two domains, and it is thought that GDP release is effected by receptor-induced opening of the cleft. Comparative analysis of the crystal structure of inactive (GDP-bound) to that of activated (GTPγS- or AlF_4^--bound) α-subunits demonstrates that the active conformation is attained by the movement of three regions (named switch 1, 2, and 3). The movement of switches 1 and 2 upon GTP binding is in direct response to the presence of the γ-phosphate group. Switch 3 has no direct contact with bound guanine nucleotide. Upon activation, switches 2 and 3 move toward each other and form multiple interactions. In transducin, switch 3 residues were shown to be critical for effector activation, possibly by conformational coupling with switch 2 (13). Switch 3 may also make contacts with the helical domain, which are important for high affinity guanine nucleotide binding (10).

Albright hereditary osteodystrophy (AHO) is an autosomal dominant disorder characterized by obesity, short stature, subcutaneous ossifications, and, in some cases, mental retardation. Most cases are associated with heterozygous inactivating

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[1] The abbreviations used are: G protein, guanine nucleotide binding protein; G_s, stimulatory G protein; $G_s\alpha$, G_s α-subunit; $G_s\alpha$ R258W, $G_s\alpha$ mutant with Arg^{258} to tryptophan substitution; $G_s\alpha$ R258A, $G_s\alpha$ mutant with Arg^{258} to alanine substitution; AHO, Albright hereditary osteodystrophy; PPHP, pseudopseudohypoparathyroidism; DTT, dithiothreitol; AlF_4^-, mixture of 10 μM $AlCl_3$ and 10 mM NaF; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR.

mutations of the gene encoding $G_s\alpha$ (14, 15). Within AHO kindreds, patients may have the somatic features of AHO alone (termed pseudopseudohypoparathyroidism (PPHP)) or AHO in association with resistance to multiple hormones that activate G_s-coupled signaling pathways (termed pseudohypoparathyroidism type Ia). While most mutations associated with AHO are frameshift deletions or splice junction mutations, some encode missense mutations that have specific effects on the functional properties of the $G_s\alpha$ protein. Examples include mutations that affect receptor coupling (16), guanine nucleotide binding (17, 18), and activation (19).

In the present report, we describe a novel $G_s\alpha$ missense mutation from an AHO patient in which an arginine residue in switch 3 (Arg^{258})[2] is substituted with tryptophan. Arg^{258} is a nonconserved residue adjacent to a highly conserved glutamic acid residue (Glu^{259}) that is important for contact between switch 2 and 3 in the activated state (7, 12). We present evidence that substitution of Arg^{258} leads to defective GDP binding, resulting in increased thermolability and decreased activation by AlF_4^-. This mutation also leads to decreased receptor activation. In the crystal structure of $G_s\alpha$ in the active state, Arg^{258} associates with a residue (Gln^{170}) located between the αD and αE helices of the helical domain, forming a "lid" over the guanine nucleotide binding pocket. Mutation of Arg^{258} is predicted to disrupt these interactions as well as an interaction between Asp^{173} in the helical domain and Lys^{293} in the GTPase domain previously shown to be important for receptor- and AlF_4^--induced activation (20). Analysis of this mutation suggests that switch 3 residues, in addition to being involved in the activation mechanism, are also involved in maintaining the basal state (*i.e.* sustaining GDP in the guanine nucleotide binding site).

EXPERIMENTAL PROCEDURES

Patient—The patient was a 24-year old white male with a diagnosis of AHO and PPHP. His birth weight was 6 pounds, 11 ounces. By age 10 months, developmental delay, brachycephaly, and decreased muscle tone were noted. Throughout childhood he was small for his age and had a stocky appearance. By 6 years, learning disabilities as well as impulsive and aggressive behavior were noted, and over the past 2 years the patient has demonstrated increased compulsive behavior. During evaluation at NIH in 1995, the patient was noted to have short stature (62 inches), moderate obesity, and a rounded face with mildly depressed nasal bridge. The patient had brachydachtyly involving the distal phalanx of the first digit and the fourth metacarpals bilaterally and intracranial calcifications in the globus pallidus. Laboratory evaluation revealed no evidence for resistance to parathyroid hormone or thyrotropin. Both of the patient's parents as well as his two sisters are clinically unaffected. The patient consented to protocols approved by the NIDDK/NIAMS institutional review board.

Preparation of Erythrocyte Membranes and Determination of G_s Activity and $G_s\alpha$ Expression—Erythrocyte membranes were prepared from the patient, his parents, and three normal subjects as described previously (21) and frozen in aliquots at −70 °C. Erythrocyte membranes were reconstituted with membranes from mutant cyc^- S49 mouse lymphomas cells (which lack $G_s\alpha$ expression), and adenylyl cyclase activity in the presence of isoproterenol (10 μM) and GTP (10 μM) was determined as described previously (21). Protein was determined by the method of Bradford (Bio-Rad). Immunoblots were performed using affinity-purified RM antibody (2 μg/ml), directed to the carboxyl-terminal decapeptide of $G_s\alpha$ (22), and bands were quantified with a PhosphorImager (Molecular Dynamics, Inc.) as described previously (18).

Nucleic Acid Isolation and Genetic Analysis—Genomic DNA was isolated from whole blood and screened for mutations within the gene encoding $G_s\alpha$ (*GNAS1*) using PCR and temperature gradient gel electrophoresis (15, 23). PCR fragments were purified using Centricon 100 filters (Amicon) and directly sequenced using the Sequenase kit (U.S. Biochemical Corp.). RNA was isolated from 100-μl aliquots of whole blood, and RT-PCR was performed as described previously (18). For amplification of the *GNAS1* exon 10 coding region, the primers were as follows: upstream, 5′-ATGTTTGACGTGGGTGGCCAGC-3′; downstream, 5′-CACAGAGATGGTGCGCAGCCAT-3′ (24). The mutation was confirmed by digestion of PCR and RT-PCR products with the restriction enzyme *Msp*I.

Construction of $G_s\alpha$ Plasmids and in Vitro Transcription/Translation—To generate $G_s\alpha$ R258W, RT-PCR fragments amplified from the patient's RNA were digested with *Hin*cII and *Sse*8387I and ligated into the transcription vector pBluescript II SK (Stratagene, La Jolla, CA) that contained wild type human $G_s\alpha$ cDNA (splice variant $G_s\alpha$-1, Ref. 24) from which the same *Hin*cII-*Sse*8387I restriction fragment had been removed. A control plasmid was created using the identical approach with material obtained from a normal control. $G_s\alpha$ R258A (CGG to GCG) was generated by PCR using a mutagenic primer. The primers were as follows (1 μM each): upstream, 5′-GACAAAGTCAACTTCCACATGTTTGACGTGGGTGGCCAGCGCGATGAACG-3′; downstream (mutagenic), 5′-GAGCCTCCTGCAGGCGGTTGGTCTGGTTGTCCTCCGCGATGACCATGTTG-3′. The DNA template was linearized vector containing the wild type $G_s\alpha$ cDNA (0.2–1 μg/ml). The PCR mixtures were denatured at 94 °C for 1 min, annealed at 65 °C for 1 min, and extended at 72 °C for 30 s for 20 cycles. PCR products were digested with *Hin*cII and *Sse*8387I and ligated into the pBluescript II SK containing $G_s\alpha$ as described above. Mutations were verified by DNA sequencing, and synthesis of full-length $G_s\alpha$ from each construct was confirmed by immune precipitation of *in vitro* translated products with RM antibody. *In vitro* transcription/translation was performed on $G_s\alpha$ plasmids as described previously (18) using the TNT coupled transcription/translation system from Promega, with the exception that in most experiments no RNase inhibitor was added. We have found that deletion of this component reduces nonspecific initiation from downstream Met codons with no detectable loss of full-length $G_s\alpha$.

Adenylyl Cyclase Assays—Wild type and mutant $G_s\alpha$ *in vitro* transcription/translation products (10 μl of translation medium) were reconstituted into 25 μg of purified S49 cyc^- plasma membranes and tested for stimulation of adenylyl cyclase in the presence of various agents as indicated in Table I (18, 25). Reactions were incubated for 15 min at 30 °C, and the amount of [^{32}P]cAMP produced was measured as described previously (26). Background activities were determined from mock transcription/translation reactions and were subtracted for final presentation of the data. Data were normalized to the relative amount of $G_s\alpha$ synthesis as determined by quantitation of [^{35}S]methionine-labeled *in vitro* transcription/translation products.

Trypsin Protection Assays—Limited trypsin digestion of *in vitro* translated $G_s\alpha$ was performed as described previously (18). Briefly, 1 μl of *in vitro* translated [^{35}S]methionine-labeled $G_s\alpha$ was incubated in incubation buffer (20 mM HEPES, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT) with or without 100 μM GTPγS or 10 mM NaF/10 μM $AlCl_3$ at various temperatures for 1 h and then digested with 200 μg/ml tosylphenylalanyl chloromethyl ketone-treated trypsin for 5 min at 20 °C. In some experiments, GDP was also included in the preincubation. Reactions were terminated by boiling in Laemmli buffer. Digestion products were separated on 10% SDS-polyacrylamide gels, and the amount of 38-kDa protected fragment was measured by PhosphorImager analysis. The percentage of protection is the signal in the 38-kDa protected band divided by the signal in the undigested full-length $G_s\alpha$ band times 100. For experiments examining the time course of GDP release, [^{35}S]methionine-labeled *in vitro* translates were incubated with 2 mM GDP for 1 h at 30 °C; chilled on ice and diluted 20-fold into incubation buffer with 100 μM GTPγS; and then transferred to a 30 °C water bath. At the indicated times, aliquots were removed, and trypsin and GDP were added to attain final concentrations of 200 μg/ml and 1 mM, respectively. For the zero time point, trypsin was added prior to transfer to 30 °C. After the addition of trypsin, the samples were immediately placed in an ice water bath and incubated for 1 h. GTPγS binds to a negligible degree within 1 h at 0 °C (data not shown).

Sucrose Density Gradient Centrifugation—[^{35}S]methionine-labeled $G_s\alpha$ was synthesized, and rate zonal centrifugation was performed on linear 5–20% sucrose gradients (200 μl) as described previously (18, 27). Gradients were prepared in 20 mM HEPES, pH 8.0, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 0.1% Lubrol-PX, and additions as described in the figure legend. 6-μl fractions were obtained and analyzed by SDS-polyacrylamide gel electrophoresis, and the relative amount of $G_s\alpha$ in each fraction was quantified as described previously (18). G$\beta\gamma$ was isolated from bovine brain (28).

Expression and Purification of $G_s\alpha$ from E. coli—Plasmid pQE60 containing the long form of bovine $G_s\alpha$ cDNA with a hexahistidine extension at the carboxyl terminus was a generous gift of A. G. Gilman

[2] All numbering is based on the $G_s\alpha$-1 sequence reported by Kozasa *et al*. (24).

and R. K. Sunahara. The Arg^{258} residue was mutated by site-directed mutagenesis using the Quickchange kit (Statagene). After mutagenesis, each cDNA was sequenced to confirm the presence of the desired mutation and to rule out PCR artifacts. After transformation into *E. coli* strain JM109, cultures were grown, $G_s\alpha$ expression was induced, and cleared lysates were prepared as described previously (29). His-tagged $G_s\alpha$ proteins were purified on 2.5-ml Ni^{2+}-nitrilotriacetic acid resin columns (Qiagen) equilibrated with TβP buffer (50 mM Tris-HCl, pH 8.0, 20 mM β-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride). Cleared lysates were loaded onto each column, and then each column was washed with 25 ml of TβPG (TβP buffer supplemented with 50 μM GDP) containing 500 mM NaCl, followed by 40 ml of TβPG containing 50 mM NaCl and 10 mM imidazole. $G_s\alpha$ was eluted with TβPG containing 50 mM NaCl, 150 mM imidazole, and 10% glycerol and then exchanged into 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 mM DTT, 50 μM GDP, and 10% glycerol and stored at −80 °C at greater than 1.5 mg/ml.

Guanine Nucleotide Binding Assays—Assays measuring the rate of binding of GTPγS were performed as described previously (30). Briefly, 25 nM purified $G_s\alpha$ was incubated with 1 μM [^{35}S]GTPγS (~30,000 cpm/pmol) in 25 mM HEPES, pH 8.0, 1 mM EDTA, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.01% Lubrol-PX in a final volume of 2 ml. At various times, 50-μl aliquots were removed and diluted with 2 ml of ice-cold stop solution (25 mM Tris-HCl, 100 mM NaCl, 25 mM $MgCl_2$, and 100 μM GTP) and maintained on ice until all samples were collected. Samples were then filtered under vacuum through nitrocellulose filters (Millipore Corp.), washed twice with 10 ml of stop solution without GTP, and dissolved in 10 ml of scintillation mixture. k_{app} values for GTPγS binding were calculated using GraphPad Prism software.

RESULTS

Identification of $G_s\alpha$ Biochemical and Genetic Defect in an AHO Patient—The patient had somatic features of AHO but no evidence of hormone resistance. G_s bioactivity in the patient's erythrocyte membranes measured by the cyc^- reconstitution assay in the presence of isoproterenol (10 μM) and GTP (10 μM) was 52% of that present in normal subjects. $G_s\alpha$ expression in these same membranes was 50% of normal based upon quantitative immunoblotting. The patient's mother and father, who do not have clinical evidence of AHO and who do not have a *GNAS1* mutation (see below) showed no decrease in G_s bioactivity or $G_s\alpha$ expression (data not shown). The *GNAS1* gene was screened for mutations by temperature gradient gel electrophoresis analysis of PCR-amplified genomic DNA fragments encompassing *GNAS1* exons 2–13 and their intron-exon splice junctions (15, 24). Temperature gradient gel electrophoresis analysis of a genomic fragment encompassing *GNAS1* exons 10 and 11, which was amplified from the patient's genomic DNA, revealed abnormally migrating bands that were not present in either parent's sample or in numerous other normal control or patient samples (data not shown). By direct sequencing of the genomic DNA fragment (Fig. 1), the patient was shown to have a heterozygous single base substitution (C to T) within the coding region of exon 10 that encodes the substitution of tryptophan for arginine at codon 258 ($G_s\alpha$ R258W). Arg^{258} is within the switch 3 region of $G_s\alpha$ (7). This mutation destroys an *Msp*I restriction site. Digestion of PCR-amplified genomic DNA fragments with *Msp*I confirmed the presence of the mutation in the patient and its absence in either parent (data not shown). This therefore represents a *de novo* mutation within this kindred.

RNA from whole blood was isolated, and the relative expression of the mutant and wild type allele in the patient was assessed by analysis of RT-PCR products spanning exon 10. A significant portion of the RT-PCR product amplified from RNA of the affected patient was resistant to *Msp*I digestion, demonstrating that the mRNA expression of mutant and wild type alleles was similar (Fig. 2). In contrast, as noted above, the expression of $G_s\alpha$ protein in erythrocyte membranes from the patient is only about 50% of that from normal membranes, suggesting that the mutant protein is probably not present in the membrane.

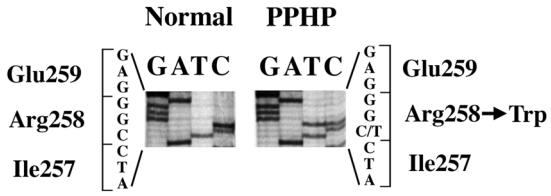

FIG. 1. **Direct sequencing of the exon 10 and 11 genomic fragment.** Genomic DNA was amplified by PCR and directly sequenced. The oligonucleotide primers used for PCR were 5′-AAGAATTCTTAGGGATCAGGGTCGCTGCTC-3′ (upstream primer) and 5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCCATGAACAGCCAGCAAGAGTGGA-3′ (downstream primer with GC clamp) with the underlined sequences complementary to the *GNAS1* gene (15, 24). Whereas the direct sequence of genomic DNA amplified from a normal subject (*left*) reveals only a CGG triplet at codon 258 (arginine), the corresponding sequence in the affected patient (PPHP, *right*) reveals a T and C at the third position of the codon. This indicates a heterozygous single base substitution that encodes the substitution of tryptophan (TGG) for arginine (CGG) at codon 258 (24).

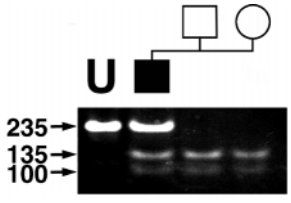

FIG. 2. ***Msp*I digestion of RT-PCR products.** A 235-base pair RT-PCR product spanning exon 10 was amplified from whole blood RNA. The products were digested with *Msp*I and electrophoresed on a 6% polyacrylamide gel. The pedigree is shown *above*, with the *filled square* representing the affected patient and the *white square* and *circle* representing the proband's father and mother, respectively. The fragment size in base pairs is indicated on the *left*. The *left lane* contains undigested RT-PCR product (*U*). The patient has 235-, 135-, and 100-base pair bands, demonstrating the presence of mutant (undigested) and wild type (digested) products. Both parents demonstrate complete *Msp*I digestion, since they lack the mutation.

Substitution of $G_s\alpha$ Arg^{258} Leads to Decreased Activation by Activated Receptor or AlF_4^-—$G_s\alpha$ R258W was cloned into the transcription vector pBluescript, and the *in vitro* transcription/translation products were compared with those of wild type $G_s\alpha$ in various biochemical assays. Since the presence of an amino acid with a bulky hydrophobic side chain (tryptophan) may introduce nonspecific steric effects, we also generated and analyzed an additional mutant in which Arg^{258} was replaced by alanine ($G_s\alpha$ R258A). After reconstitution of translation products into purified S49 cyc^- membranes, each mutant was efficient at stimulating adenylyl cyclase in the presence of GTPγS, with the response from $G_s\alpha$ R258W about the same as and that from $G_s\alpha$ R258A slightly greater than that of wild type $G_s\alpha$ (see Table I). In contrast, both mutants had markedly decreased ability to stimulate adenylyl cyclase in the presence of isoproterenol or AlF_4^- (Table I). Therefore, substitution of Arg^{258} leads to specific defects in activation by activated receptor or AlF_4^-.

We next examined the ability of AlF_4^- or GTPγS to protect each mutant from trypsin digestion, which measures the ability of each agent to bind to $G_s\alpha$ and induce the active conformation (31). In the inactive, GDP-bound state, two arginine residues within switch 2 (most likely Arg^{228} and Arg^{231}, based upon sequence homology with transducin) are sensitive to trypsin digestion, leading to the generation of small molecular weight fragments. When $G_s\alpha$ attains the active conformation, these residues are inaccessible to trypsin digestion (7); therefore, trypsinization of activated $G_s\alpha$ generates a partially protected 38-kDa product. Wild type $G_s\alpha$ was well protected by AlF_4^- or GTPγS at temperatures up to 37 °C (Fig. 3, Table II).

TABLE I
Adenylyl cyclase stimulation by $G_s\alpha$ mutants

In vitro transcription/translation products were mixed with purified cyc$^-$ membranes and assayed for adenylyl cyclase stimulation as described under "Experimental Procedures." Results are expressed as the mean ± S.D. (σ_{n-1}) of triplicate determinations and are corrected for the relative level of synthesis of each mutant to wild type. $G_s\alpha$ R258W and $G_s\alpha$ R258A were synthesized to 86 ± 17% (n = 16) and 105 ± 25% (n = 6) of wild type $G_s\alpha$ levels as determined by *in vitro* translation with [^{35}S]methionine, SDS-polyacrylamide gel electrophoresis, and PhosphorImager analysis. Background values determined from mock transcription/translation reactions (in pmol of cAMP/ml of translation medium/15 min as follows: GTP, 29 ± 1; isoproterenol, 39 ± 5; GTPγS, 39 ± 2; and AlF_4^-, 64 ± 6) were subtracted from each determination. Values are shown in pmol of cAMP/ml of translation product/15 min, and the percentage of the wild type value is shown in parentheses.

$G_s\alpha$ mutation	GTP (100 μM)	Isoproterenol (10 μM) + GTP (100 μM)	GTPγS (100 μM)	AlF_4^-[a]
Wild type	20 ± 6	231 ± 6	167 ± 7	359 ± 41
R258W	24 ± 5	73 ± 9 (32 ± 4)	155 ± 7 (93 ± 6)	177 ± 12 (49 ± 7)
R258A	8 ± 1	56 ± 7 (24 ± 3)	221 ± 4 (132 ± 6)	223 ± 19 (62 ± 9)

[a] 10 mM NaF, 10 μM $AlCl_3$, and 100 μM GDP.

At 37 °C, GTPγS was able to protect $G_s\alpha$ R258A and $G_s\alpha$ R258W to levels of 85 and 68% of wild type $G_s\alpha$, respectively (Fig. 3, Table II). Consistent with the results of the cyc$^-$ reconstitution assays, AlF_4^- was less effective than GTPγS in protecting either mutant from trypsin digestion at higher temperatures, with $G_s\alpha$ R258W being more severely affected than $G_s\alpha$ R258A (Fig. 3, Table II). AlF_4^- protected $G_s\alpha$ R258A normally at 25 and 30 °C but only about 40% as well as wild type $G_s\alpha$ at 37 °C. For $G_s\alpha$ R258W, trypsin protection by AlF_4^- was 70, 59, and 6% of wild type $G_s\alpha$ at 25, 30, and 37 °C, respectively. These data demonstrate that both mutants are capable of attaining the activated conformation with GTPγS or AlF_4^- (although for AlF_4^- more efficiently at lower temperatures). The somewhat decreased trypsin protection of $G_s\alpha$ R258W in the presence of GTPγS at 37 °C or AlF_4^- at lower temperatures may be due to steric effects resulting from the bulky tryptophan side chain.

Substitution of $G_s\alpha$ Arg^{258} Leads to Decreased Affinity for GDP—GTPγS was a more effective activator of both mutants than AlF_4^- at 37 °C. It is possible that both mutants have decreased affinity for AlF_4^- or activate poorly when GDP and AlF_4^- are bound. Another possibility is that at higher temperatures the mutants bind GDP more poorly, which would result in decreased activation by AlF_4^-, since GDP binding is a prerequisite for AlF_4^- binding and activation. To address these possibilities, the effect of increasing the concentration of GDP on AlF_4^--induced trypsin protection of $G_s\alpha$ R258W and R258A was determined[3] (Fig. 3, Table II). In the presence of increased GDP concentrations, the ability of each mutant to be protected by AlF_4^- was partially or fully restored to normal. The effect was dose-dependent with maximum protection attained at a GDP concentration of 2 mM (data not shown). These data suggest that decreased protection of the mutants by AlF_4^- is not due to a specific defect in AlF_4^- binding but rather to decreased ability of the mutants to maintain the GDP-bound state at higher temperatures. GTP was equally effective in enhancing protection in the presence of AlF_4^- (data not shown). In the absence of AlF_4^-, no protection was observed with either GDP or GTP. The latter observation suggests that the mutants have intact GTPase function.

We next indirectly determined the relative rate of GDP release in the inactive state from both mutants and wild type

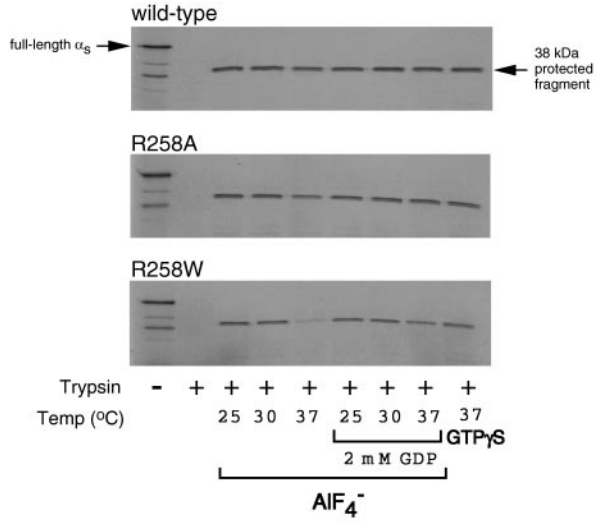

FIG. 3. **Trypsin protection of *in vitro* translated $G_s\alpha$ R258W and R258A in the presence of GTPγS or AlF_4^-.** *In vitro* translates were digested with tosylphenylalanyl chloromethyl ketone-treated trypsin (200 μg/ml) for 5 min at 20 °C after 1-h preincubations at various temperatures and in the presence of various agents indicated *below*. For each form of $G_s\alpha$, the full-length undigested $G_s\alpha$ is shown in the *far left lane*, and complete digestion in the absence of activators is demonstrated in the *next lane*. Digestion of wild type $G_s\alpha$ in the presence of either AlF_4^- or GTPγS (100 μM) produced a 38-kDa protected band at all temperatures examined (for GTPγS, only the results at 37 °C are shown, *far right*). On shorter exposures, it is clear that the 38-kDa protected band is actually a doublet (not shown). GTPγS was able to protect both mutants at all temperatures. For $G_s\alpha$ R258A, protection by AlF_4^- was normal at 30 °C and somewhat decreased at 37 °C, while for $G_s\alpha$ R258W, protection by AlF_4^- was somewhat decreased at 30 °C and markedly decreased at 37 °C. For both mutants, the addition of excess GDP (2 mM) to the preincubation was able to partially or fully restore protection by AlF_4^-. Quantitation of trypsin protection assays is presented in Table II.

$G_s\alpha$. [^{35}S]methionine-labeled *in vitro* translation products were preincubated with 2 mM GDP and then diluted 20-fold into a solution with 100 μM GTPγS, and the level of trypsin protection was determined at various time points (Fig. 4). Since the rate of GTPγS binding and activation is limited by the rate of GDP release, the rate of increase of trypsin protection is a function of the rate of GDP release (17, 32, 33). Wild type $G_s\alpha$ reached maximum protection by 8–10 min, whereas for both $G_s\alpha$ R258A and R258W equal maximal protection was achieved by 2 min (the first time point examined). These data suggest that the rate of GDP release from the mutants in the inactive state is at least 4–5 times greater than the rate of GDP release from wild type $G_s\alpha$.

To confirm these observations and to more accurately determine the relative rates of GDP release, we expressed and purified bovine wild type $G_s\alpha$ and $G_s\alpha$ R258A from *E. coli* and

[3] We estimate that the carryover guanine nucleotide from the *in vitro* transcription/translation reaction into the trypsin protection assays is 50–100 μM, based on the concentrations required to support coupled transcription and translation reported by Craig *et al.* (40). This concentration is well above the the K_d of $G_s\alpha$ for guanine nucleotides (<1 μM) (41). The distribution between GDP and GTP is unknown, but presumably more GTP would be present due to the nucleotide-regenerating system present in the translation reaction. However, we have determined that GTP is equally effective in the trypsin protection assay when AlF_4^- is used (data not shown). The carryover into the samples loaded onto sucrose gradients (Fig. 6) is estimated to be 240–320 μM.

TABLE II
Influence of excess GDP on AlF_4^--induced trypsin protection

Temperature	Treatment	Wild type	R258W[a]	R258A[b]
		% protection	*% of wild type*	*% of wild type*
25 °C	AlF_4^-	60 ± 4	70 ± 6	107 ± 10
	AlF_4^- + 2 mM GDP	66 ± 5	67 ± 2	104 ± 8
30 °C	AlF_4^-	67 ± 4	59 ± 5	87 ± 10
	AlF_4^- + 2 mM GDP−	66 ± 4	64 ± 3	85 ± 3
37 °C	AlF_4^-	48 ± 2[c]	6 ± 3[c,d]	40 ± 8[c,d]
	AlF_4^- + 2 mM GDP	57 ± 5	48 ± 3[c]	78 ± 4
	100 μM GTPγS	58 ± 3	68 ± 4	85 ± 3

These data were obtained from four experiments of the type presented in Fig. 3. The amount of the 38-kDa trypsin-stable $G_s\alpha$ fragment was determined by PhosphorImager analysis and for wild type $G_s\alpha$ is expressed as a percentage of undigested $G_s\alpha$ (mean ± S.E.). No protection was observed when AlF_4^- and GTPγS were excluded. Maximum trypsin protection has a theoretical limit of 71%, based on the removal of 2 of 7 total methionine residues by trypsin. For $G_s\alpha$ R258W and R258A, the data are expressed as percentage of wild type under each condition (mean ± S.E.).

[a] The percentage protection of $G_s\alpha$ R258W was significantly less than that of wild type $G_s\alpha$ under all conditions (Student's *t* test).

[b] The percentage protection of $G_s\alpha$ R258A was significantly less than that of wild type $G_s\alpha$ at 37 °C with all agents and at 30 °C in the presence of AlF_4^- and GDP (Student's *t* test).

[c] $p < 0.05$ *versus* GTPγS (Student's *t* test).

[d] $p < 0.05$ *versus* AlF_4^- + GDP (Student's *t* test).

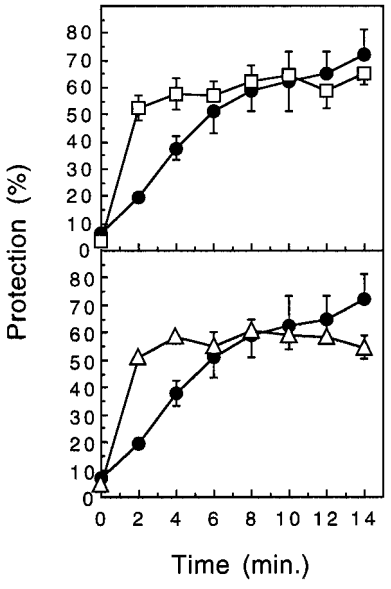

FIG. 4. **Time course of GTPγS-induced trypsin resistance.** *In vitro* translates were incubated with 2 mM GDP for 1 h at 30 °C and then chilled on ice and diluted 20-fold into a solution containing 100 μM GTPγS and then transferred to a 30 °C water bath. At the indicated times, aliquots were removed and mixed with a solution of tosylphenylalanyl chloromethyl ketone-treated trypsin and GDP (final concentrations, 200 μg/ml and 1 mM, respectively). For the zero time point trypsin was added prior to transfer to 30 °C. After the addition of trypsin, the samples were immediately placed in an ice water bath and incubated for 1 h. GTPγS binds to a negligible degree within 1 h at 0 °C. Trypsin digestion was terminated, and the percentage of trypsin protection was determined as described under "Experimental Procedures." The data presented are the mean ± S.D. (σ_{n-1}) of three independent experiments. The data for wild type $G_s\alpha$ (●) are the same in the *top* and *bottom panels*. The data for $G_s\alpha$ R258A (□, *top panel*) and $G_s\alpha$ R258W (△; *bottom panel*) were separated for clarity. For both mutants, the apparent rate of GDP release (equivalent to the rate of increase of trypsin protection in the presence of GTPγS) was at least 4–5 times the rate for wild type $G_s\alpha$.

directly measured the apparent on rate of GTPγS. The rate of GTPγS binding has been shown to be limited by the rate of GDP dissociation, and the experimentally determined values of these two rates are essentially identical (32, 33). This assay has also been previously used as a measure of the GDP dissociation

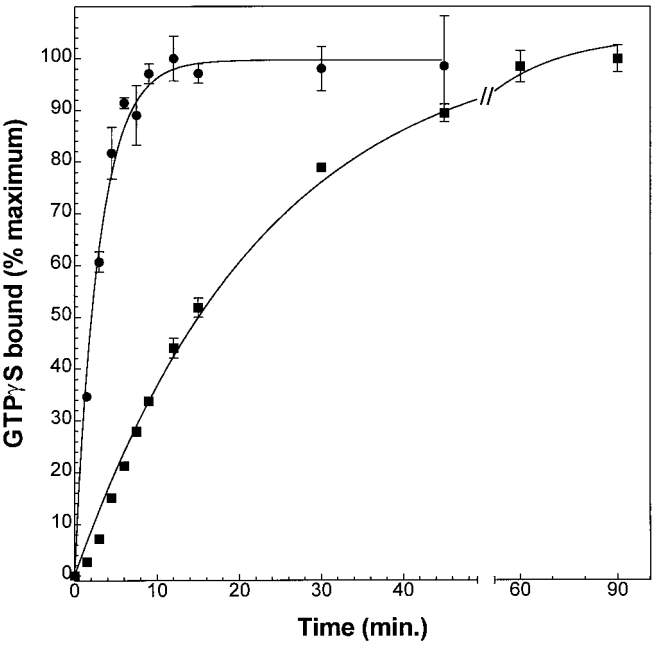

FIG. 5. **Time course of GTPγS binding to purified $G_s\alpha$.** Bovine wild type $G_s\alpha$ and $G_s\alpha$ R258A with carboxyl-terminal hexahistidine extension were expressed and purified from *E. coli*, and the rate of GTPγS binding for each was determined. Wild type $G_s\alpha$ (■) and $G_s\alpha$ R258A (●) were incubated in 1 μM [^{35}S]GTPγS (~30,000 cpm/pmol) at 20 °C in 25 mM HEPES, pH 8.0, 1 mM EDTA, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.001% Lubrol-PX. At the indicated times, the reaction was terminated, and bound GTPγS was determined as described under "Experimental Procedures." Data were fit ($r^2 > 0.995$) to the equation $B = B_{max}(1 - e^{-kt})$, where B represents GTPγS bound at time t, B_{max} represents maximal GTPγS bound, and k represents the apparent on rate. Each point is the mean ± S.D. of triplicate determinations. The B_{max} values were 3.0 pmol for wild type $G_s\alpha$ and 1.3 pmol for $G_s\alpha$ R258A. k_{app} values averaged from three independent experiments were 0.04 ± 0.00 min^{-1} for wild type $G_s\alpha$ and 0.36 ± 0.02 for $G_s\alpha$ R258A.

rate in another $G_s\alpha$ mutant (17). The k_{app} for GTPγS binding was 0.04 min^{-1} for wild type $G_s\alpha$ *versus* 0.36 min^{-1} for $G_s\alpha$ R258A, indicating a 10-fold increased rate of GTPγS binding (or GDP dissociation) to $G_s\alpha$ R258A compared with wild type $G_s\alpha$ (Fig. 5). Under the same experimental conditions, the k_{app} values of GTPγS binding to $G_s\alpha$ R258A do not increase linearly with increasing concentrations of GTPγS, indicating that GDP was initially bound to the protein (32). Although the k_{app} for wild type $G_s\alpha$ that we determined was less than that determined for the long form of $G_s\alpha$ in one study (0.34 min^{-1}; Ref. 34), it was similar to that reported in another study for an amino-terminal hexahistidine-tagged long form of $G_s\alpha$ (0.05 min^{-1}; Ref. 35). We attempted to measure the binding affinities for GDP by a competition assay with [^{35}S]GTPγS (36). Although the results appeared consistent with $G_s\alpha$ R258A having reduced GDP affinity, the interpretation is complicated by evidence for a heterogeneous population of $G_s\alpha$ R258A molecules with varying affinities for GDP (data not shown).

Decreased GDP Binding by $G_s\alpha$ Arg^{258} Mutants Increases Their Thermolability—Since the rate of GDP release from the $G_s\alpha$ Arg^{258} mutants is faster than that from wild type $G_s\alpha$, we would predict that a greater proportion of each mutant exists in the guanine nucleotide-free state. Other $G_s\alpha$ mutants with decreased affinity for guanine nucleotide have been shown to have increased thermolability (17, 18), presumably since the guanine nucleotide-free state is unstable. To examine the thermostability of $G_s\alpha$ R258A and R258W, we analyzed their distribution within sucrose gradients after preincubation at various temperatures for 1 h in the presence or absence of 2 mM

GDP[3] or 100 μM GTPγS (Fig. 6*A*). When *in vitro* translates of $G_s\alpha$ R258A and R258W were held on ice, the gradient profile was virtually the same as that of wild type $G_s\alpha$ and consistent with the overall proper conformation (sedimentation coefficient ~3.7 S; Ref. 18). When preincubated on ice with purified bovine brain $\beta\gamma$, the sedimentation coefficient of both mutants and wild type $G_s\alpha$ increased from 3.7 to 5.0 S (Fig. 6*B*), demonstrating that at low temperatures each mutant maintained the ability to interact with $\beta\gamma$ (18). Similar results were obtained with $G_s\alpha$ R258W after incubation with $\beta\gamma$ at 30 °C (data not shown).

When preincubated for 1 h at 30 °C, some of the $G_s\alpha$ R258W protein was present in a ~6.3 S or higher peak, presumably due to denaturation and aggregation, while the profiles for $G_s\alpha$ R258A and wild type $G_s\alpha$ were the same as after the 0 °C preincubation. After preincubation for 1 h at 37 °C, both mutants displayed a more severe pattern of aggregation, with most of each located in the latter half of the gradient with sedimentation values greater than 7.2 S. This pattern is most likely the direct result of denaturation, although we have not conclusively proven that this material in fact represents denatured $G_s\alpha$. Wild type $G_s\alpha$, although showing some loss of the native 3.7 S peak, was significantly more stable than either mutant at 37 °C. When 2 mM GDP or 100 μM GTPγS was included in the preincubation, $G_s\alpha$ R258A (as well as wild type $G_s\alpha$) was fully protected from the denaturing effects of mild heat treatment (37 °C). Denaturation of $G_s\alpha$ R258W at 37 °C was mostly prevented by 100 μM GTPγS and to a somewhat lesser extent by 2 mM GDP. AlF_4^- provided no stabilization of $G_s\alpha$ R258W above that observed with 2 mM GDP, whereas AlF_4^- alone was sufficient to stabilize wild type completely against denaturation at 37 °C (data not shown). These results suggest that both $G_s\alpha$ R258W and R258A are more thermolabile than wild type $G_s\alpha$ and that this is due to decreased affinity for guanine nucleotides, since the addition of guanine nucleotides can partially or fully reverse denaturation of these mutants at higher temperatures. For $G_s\alpha$ R258W, decreased protection by AlF_4^- and increased thermolability were observed at both 30 and 37 °C, while for $G_s\alpha$ R258A these abnormalities were only observed at 37 °C. For both mutants, these defects were reversed by the addition of excess GDP. It therefore appears that both mutants have a similar underlying biochemical abnormality (decreased GDP binding) but that the abnormality is more severe in $G_s\alpha$ R258W than in $G_s\alpha$ R258A.

DISCUSSION

We identified a heterozygous missense mutation ($G_s\alpha$ R258W) in a patient with AHO and PPHP. This mutation, as well as a mutation that replaces Arg^{258} with alanine ($G_s\alpha$ R258A), encodes a $G_s\alpha$ protein with impaired function. Both mutants stimulated adenylyl cyclase and attained the active conformation normally in the presence of GTPγS. However, their ability to stimulate adenylyl cyclase in the presence of AlF_4^- or activated receptor (isoproterenol plus GTP) was significantly attenuated. Consistent with this result, GTPγS was better able than AlF_4^- to protect both mutants from trypsin digestion. Excess GDP was able to partially or fully restore AlF_4^- protection, suggesting that decreased activation by AlF_4^- was due to decreased binding of GDP in the inactive state. Both mutants were shown to have a markedly increased rate of guanine nucleotide turnover, reflecting an increased rate of GDP release. Defective guanine nucleotide binding in these mutants probably results from disruption of interactions between the helical and GTPase domains (see below). Although the overall conformation (and ability to bind $\beta\gamma$) of both mutants was normal at lower temperatures, both denatured more rapidly at physiological temperatures. The mutants were pro-

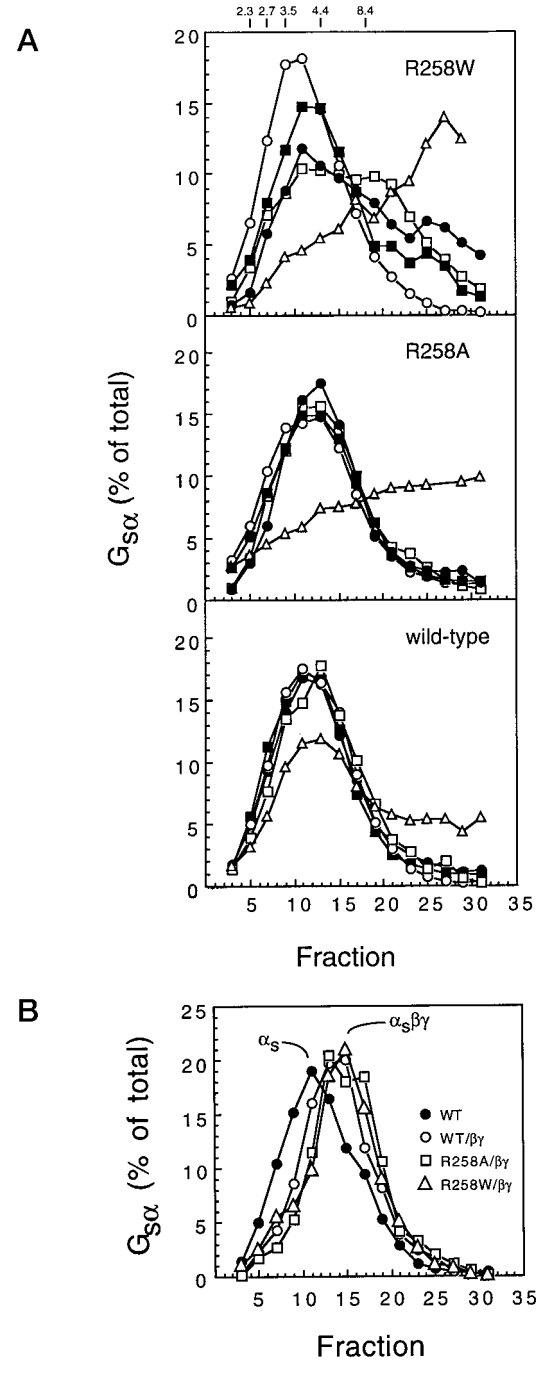

FIG. 6. **Sucrose density gradient centrifugation of $G_s\alpha$ *in vitro* translation products.** *A*, [^{35}S]methionine-labeled *in vitro* translates of both $G_s\alpha$ Arg^{258} mutants and wild type $G_s\alpha$ were preincubated for 1 h at 0 (○), 30 (□), 37 (△), or 37 °C in the presence of an added 2 mM GDP (●) or 37 °C in the presence of added 100 μM GTPγS (■). Following incubation, the samples were layered over a 200-μl 5–20% linear sucrose gradient and centrifuged for 1 h at 4 °C at 436,000 × *g*. Fractions (6 μl each) were collected, and odd numbered fractions were analyzed by SDS-polyacrylamide gel electrophoresis and PhosphorImager analysis (18). The data are expressed as the percentage of total $G_s\alpha$ present in each fraction. Fraction 1 represents the top of the gradient. For samples in which either GDP or GTPγS was included in the preincubation, it was also present in the gradient itself. The positions of the peak concentrations of standard proteins in the gradient are shown at the *top* and are as follows: soybean trypsin inhibitor, 2.3 S; carbonic anhydrase, 2.7 S; ovalbumin, 3.5 S; bovine serum albumin, 4.4 S, and phosphorylase b, 8.4 S. *B*, [^{35}S]methionine-labeled *in vitro* translates of both $G_s\alpha$ Arg^{258} mutants and wild type $G_s\alpha$ were preincubated for 1 h at 0 °C in the presence or absence of purified bovine brain G$\beta\gamma$ (20 μg/ml) and subjected to sucrose density gradient centrifugation. In the presence of $\beta\gamma$, both mutants and wild type $G_s\alpha$ shifted to a peak of ~5 S. For clarity, each mutant is shown only in the presence of $\beta\gamma$.

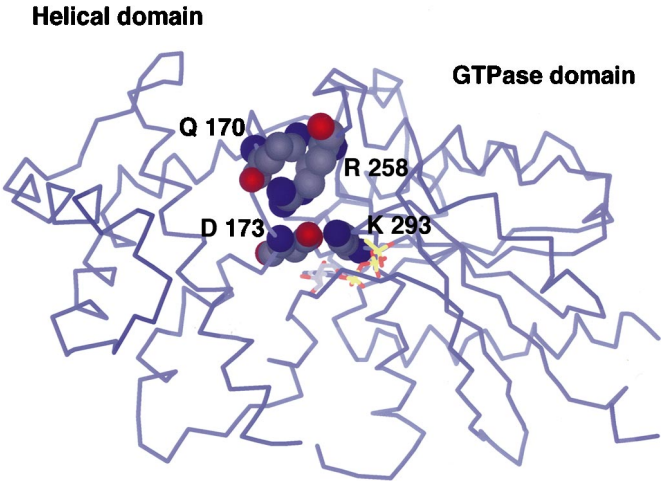

FIG. 7. **Crystal structure of $G_s\alpha$-GTPγS.** The coordinates for the short form of bovine $G_s\alpha$-GTPγS (Protein Data Bank accession code 1AZT (12)) were loaded into Look, version 3.0. The numbering corresponds to the long form of $G_s\alpha$. The atoms in residue Arg^{258} in the GTPase domain and residue Gln^{170} in the helical domain are shown as space-filled CPK spheres (carbon, *gray*; oxygen, *red*; nitrogen, *blue*; sulfur and phosphorus, *yellow*). These interactions keep a "lid" over the cleft between the two domains. Asp^{173} in the helical domain and Lys^{293} in the conserved NKXD motif of the GTPase domain, which form a salt bridge between the two domains, are also highlighted. GTPγS is shown in a *yellow* and *red* stick model, and the remainder of the protein is shown as the α-carbon trace.

tected from denaturation by excess guanine nucleotide, suggesting that increased thermolability is the direct result of decreased guanine nucleotide binding. Decreased guanine nucleotide binding is associated with increased thermolability in other $G_s\alpha$ mutations (17, 18), and it is presumed that α-subunits are unstable in the absence of bound guanine nucleotide. The expression of $G_s\alpha$ is significantly reduced in erythrocyte membranes from the affected patient and membrane targeting of $G_s\alpha$ R258W is reduced when expressed in S49 cyc^- cells (data not shown). Whether abnormal targeting is due to denaturation or more subtle conformational changes affecting $\beta\gamma$ or membrane binding is unclear. However, lack of expression of $G_s\alpha$ R258W in the membrane at physiological temperature is probably the overriding defect in the affected patient.

Although $G_s\alpha$ R258W shows a somewhat more severe phenotype than $G_s\alpha$ R258A, both essentially behave similarly, suggesting that substitution of Arg^{258} is disrupting specific interactions of this residue with other $G_s\alpha$ residues. Examination of the molecular structure of GTPγS-bound $G_s\alpha$ reveals a direct contact between Arg^{258} (in switch 3 of the GTPase domain) with a residue (Gln^{170}) in the helical domain located in the loop between αD and αE (Fig. 7). Mutation of Arg^{258} to either tryptophan or alanine would be predicted to disrupt this interaction between the GTPase and helical domains. Since interactions between Arg^{258} and the helical domain act as a "lid" over the cleft that contains the guanine nucleotide binding pocket, mutations of Arg^{258} would be predicted to open the cleft and allow greater dissociation of the bound guanine nucleotide, as observed in our experiments. Arg^{258} is not conserved among different G protein α-subunits but is conserved in $G_s\alpha$ from various species. In contrast, Gln^{170} is highly conserved, and in $G_s\alpha$ it also interacts with Val^{256}, which is conserved in most G protein α-subunits. Substitution of Arg^{258} might also perturb the interaction between Gln^{170} and Val^{256}.

In $G_s\alpha$ there is salt bridge between the side chains of Asp^{173} in the helical domain and Lys^{293} in the GTPase domain (within the conserved guanine nucleotide binding motif NKXD) (Fig. 7). Codina and Birnbaumer (20) showed that mutating either residue[4] results in normal activation by GTPγS but decreased activation by AlF_4^- or activated receptor, similar to what we observed with Arg^{258} substitutions. Since both Asp^{173} and Gln^{170} lie within the interhelical loop between αD and αE, which is in close contact with the GTPase domain, it is possible that mutations of Arg^{258} may also disturb the salt bridge between Asp^{173} and Lys^{293}, which would lead to defective activation. Repositioning of the conserved lysine in the NKXD motif could directly result in decreased guanine nucleotide binding by altering the conformation of the guanine nucleotide binding pocket.

The exact mechanism by which substitution of Arg^{258} leads to defective receptor-mediated activation is not well defined. Several regions in transducin that are critical for receptor binding and activation were identified by scanning mutagenesis, although the switch 3 region was not mutagenized in that study (37). Decreased receptor-mediated activation could be the direct result of decreased binding to $\beta\gamma$ or receptor. The Arg^{258} mutants were capable of binding to $\beta\gamma$. Li and Cerione (13) demonstrated that deletion of the whole switch 3 region of transducin had no effect on interactions with $\beta\gamma$ or its receptor (rhodopsin). Moreover, crystal structures of transducin and $G_i\alpha$ do not demonstrate direct interactions between switch 3 and $\beta\gamma$ (9, 11). Disturbance of the salt bridge between the helical and GTPase domains results in severely decreased receptor activation (see above; Ref. 20). Studies on $G_s\alpha/G_{i2}\alpha$ chimeras suggest that interactions between switch 3 residues and the helical domain are critical not only to maintain the basal state but also for receptor-mediated activation (38).

Decreased receptor activation could be the direct result of a GTP binding defect, as demonstrated by mutation of a conserved switch 2 arginine ($G_s\alpha$ R231H) in an AHO patient (19, 39). Similar to the Arg^{258} mutations, this mutation leads to normal GTPγS-mediated but decreased AlF_4^-- and receptor-mediated activation. In the GTP-bound state, Arg^{231} interacts with several residues in the α3 helix and switch 3 regions, including the conserved glutamic acid residues Glu^{259} and Glu^{268}. Disrupting these interactions presumably leads to a GTP binding defect, resulting in decreased receptor-mediated activation (19). It is of interest that mutation of Glu^{259} results in a similar phenotype.[5] In contrast to the Arg^{258} mutants, $G_s\alpha$ R231H did not appear to have a GDP binding defect. GTP alone did not protect either Arg^{258} mutant or wild type $G_s\alpha$ from trypsin protection, but it was able to restore trypsin protection of $G_s\alpha$ R258W in the presence of AlF_4^- with a similar dose response as GDP (data not shown), suggesting that GTP as well as GDP binding may be altered by substitution of Arg^{258}. While GTPγS binding might also be predicted to be decreased, it has been proposed that defective GTP binding may result in a conditional activation defect that is only obvious in states in which guanine nucleotide binding is destabilized (such as interaction with activated receptor; Ref. 19). It was postulated that decreased activation of $G_s\alpha$ R231H by AlF_4^- is due to an inability of the mutant to stably maintain the GDP-AlF_4^- complex in the guanine nucleotide binding pocket, and it was demonstrated that the complex could be stabilized by high concentrations of Mg^{2+} (19). The Arg^{258} mutants showed decreased activation by AlF_4^- even in the presence of 10 mM Mg^{2+}. Moreover the defect was corrected with high concentrations of GDP, suggesting that for $G_s\alpha$ R258W and R258A, decreased activation by AlF_4^- is due to decreased GDP binding.

In summary, genetic analysis of the gene encoding $G_s\alpha$ in an

[4] Asp^{173} and Lys^{293} are referred to as Asp^{158} and Lys^{278}, respectively, in Ref. 20, since in that paper the numbering was based upon the $G_s\alpha$-3 isoform (24).

[5] D. Warner and L. S. Weinstein, unpublished observations.

AHO patient identified a residue in the switch 3 region that is critical for normal guanine nucleotide binding and receptor activation. This residue interacts with a residue in the helical domain and underscores the importance of interdomain interactions in both guanine nucleotide binding and receptor-mediated activation. This study demonstrates that identification and analysis of $G_s\alpha$ mutations in AHO patients can further our understanding of G protein function.

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